Optimizing preservation protocols to extract high-quality RNA from different tissues of echinoderms for Next Generation Sequencing

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Abstract

Transcriptomic information provides fundamental insights into biological processes. Extraction of quality RNA is a challenging step, and preservation and extraction protocols need to be adjusted in many cases. Our objectives were to optimize preservation protocols for isolation of high-quality RNA from diverse echinoderm tissues, and to compare the utility of parameters as absorbance ratios and RIN values to assess RNA quality. Three different tissues (gonad, oesophagus, and coelomocytes) were selected from the sea urchin *Arbacia lixula*. Solid tissues were flash frozen and stored at -80ºC until processed. Four preservation treatments were applied to coelomocytes: flash freezing and storage at -80ºC, RNA*later* and storage at -20ºC, preservation in TRIzol reagent and storage at -80ºC, and direct extraction with TRIzol from fresh cells. Extractions of total RNA were performed with a modified TRIzol protocol for all tissues. Our results showed high values of RNA quantity and quality for all tissues, showing non-significant differences among them. However, while flash freezing was effective for solid tissues, it was inadequate for coelomocytes because of the low-quality of the RNA extractions. Coelomocytes preserved in RNA*later* displayed large variability in RNA integrity and insufficient RNA amount for further isolation of mRNA. TRIzol was the most efficient system for stabilizing RNA which resulted on high RNA quality and quantity. We did not detect correlation between absorbance ratios and RNA integrity. The best strategies for assessing RNA integrity was the visualization of 18S and 28S bands in agarose gels and estimation of RIN values with Agilent Bioanalyzer chips.

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Introduction

Echinoderms are keystone species which often act as ecosystem engineers and play an important role within food chains in most oceans around the world (Harrold & Redd 1985; Uthike *et al*. 2009; Wangensteen *et al.* 2011). Besides its ecological significance, this animal group has been accepted as an excellent model system in experimental science worldwide (García-Arrarás & Dolmatov 2010, Matranga *et al*. 2000, 2005, 2012).

During the last years, as a consequence of global warming, ocean acidification, and pollution increase, the study of stress response in animal species has become a pivotal subject within the scientific community (Kassahn *et al*. 2009), and echinoderms species have been again ideal models for monitoring marine environmental hazards (Dupont *et al*. 2010). They have been used for decades in the evaluation of marine pollutant's toxicity (Ozretic & Krajnovic-Ozretic 1985; Coteur *et al.* 2003; Russo *et al*. 2003; Oweson *et al.* 2008; Buono *et al*. 2012 among others), and most recently to assess the effect of ocean acidification and temperature increase from different technical approaches (see some examples in Kurihara & Shirayama 2004**;** Byrne *et al*. 2009; O'Donnell *et al.* 2009; Hernroth *et al*. 2011, Martin *et al*. 2011, Dupont *et al*. 2008, 2012). Few studies have explored the effect of environmental anomalies in cellular, biochemical, and gene expression response (Matranga *et al*. 2000, 2002; Hernroth *et al*. 2011). One of the most important aspects of stress in adult echinoderms is the immune and protective response, which has been very briefly studied, identifying only a handful of genes. Genomic information extracted from the sea urchin *Strongylocentrotus purpuratus* allowed to identify some gene families involved in the response to infections (Hibino *et al*. 2006; Rast *et al*. 2006) but transcriptomic response to environmental stressors, screening

expression level of hundred of genes, has been exclusively analyzed for larvae of only two echinoderm species so far (Todgham & Hofmann 2009; O'Donnell *et al*. 2009).

Among the diversity of tissues in echinoderms, coelomocytes have been selected as biomarkers to study stress response on adults because of their prompt response to stressors (Matranga *et al*. 2000, 2005; Pinsino *et al*. 2008). These cells, present in the coelomic fluid within the body cavity of adult echinoderms, are recognized as the immune effectors. There are, at least, four subpopulations of cells based on their structural attributes, which respond to injuries and stressors through chemotaxis, phagocytosis, encapsulation, and cytotoxicity (Gross *et al.* 1999; Matranga *et al*. 2000, 2005; Smith *et al.* 2006, 2010). They respond by the activation of a serial of genes related to the immune defense (Smith *et al.* 1996). Environmental stressors as temperature shift, UV radiation, pollutants, and pH decrease can reduce protective capacity of coelomocytes, and induce activation of the heat shock protein 70 (hsp70) expression (Hernroth *et al.* 2011; Pinsino *et al*. 2008; Matranga *et al*. 2000, 2002, 2012), but the massive sequencing of the coelomocyte transcriptome under the influence of different stressors has not been taken so far.

The rapidly decreasing costs of high throughput sequencing are currently pushing the boundaries of the applications of short reads (either from genomic or transcriptomic origin) in all fields (Collins *et al*. 2008; Riesgo *et al*. 2012a). Transcriptomic information is used in a wide range of studies and provides fundamental insights into biological processes and applications (Surget-Groba & Montoya-Burgos 2010) such as levels of gene expression (Collins *et al*. 2008; Torres *et al*. 2008), gene expression profiles after experimental treatments or infection (Hegedus *et al*. 2009), discovery of tissue biomarkers (Disset *et al*. 2009), cancer

gene expression (Morrissey *et al*. 2008), gene discovery (Hahn *et al.* 2009; Riesgo *et al*. 2012a), gene content (Reinhardt *et al*. 2009), and isolation of conserved ortholog genes for phylogenomic purposes (Smith *et al*. 2011; Dunn et al. 2008), among others. Such analyses involve complementary DNA (cDNA) library construction from total or messenger RNA of usually large numbers of samples. For some cases, the extraction of RNA proves as one of the most challenging steps of the whole library construction processes (Gayral *et al*. 2011; Riesgo *et al*. 2012b; Hillyard & Clark 2012). In these cases, the optimization of the extraction protocol is essential for ensuring the required amount of RNA (depending on the protocol) with the adequate RNA integrity, which is the main requirement for subsequent retrotranscription of RNA into cDNA. Most protocols involve some sort of preservation of the RNA, because immediate RNA extraction is not always possible. Since recently, flash-freezing of tissue or cell pellets and preservation in RNA*later* are amongst the most frequent preservation methods for animals. However, for certain tissues, those preservation strategies have proved to be sub-ideal (Riesgo *et al.* 2012b; Hillyard & Clark 2012). Therefore, protocol optimization is often crucial to ensure further procedures with critical samples.

The objectives of our study were a) to optimize preservation and storage methods to isolate high-quality RNA from different tissues of echinoderms, an animal group extensively used as a model system in research, and b) to test accuracy for two different measures of RNA quality, absorbance ratios (A260/280 and A260/230) and RIN values. This study looked at two important parameters of the RNA extraction, concentration and quality.

Material and Methods

Sample and tissue collection

Thirty two specimens of *Arbacia lixula*, one of the most common sea urchin in the Mediterranean Sea, were collected by snorkeling or SCUBA diving at Santa Anna, Blanes (41°40'22.47"N 2°48'10.81"E, Northwestern Mediterranean), and maintained in an aquarium for few hours until processed. Samples from three different tissue types: coelomocytes from coelomic fluid, gonads, and oesophagus (digestive) were collected from the sea urchins.

Coelomic fluid was withdrawn from the body cavity with sterile syringes (21-gauge needle) through an insertion in the peristomial membrane. Syringes were preloaded with 5 mL of cold anticoagulant buffer composed of 80% CM-ASW (Ca^{2+}/ Mg^{2+} free sea water, artificially made in DEPC treated water) and 20% EDTA stock solution (13.53 g/l) (see Matranga *et al*. 2012). Approximately 10 mL of the cell suspension containing 15-10 x10^6 coelomocytes cells was immediately centrifuged at 12,000 g for 6 min at 4°C, and a pellet of coelomocytes recovered. Coelomocyte cells were then preserved following four different treatments: a) flash freezing in liquid nitrogen and immediate storage at -80°C (LN_2), b) immersion in 2 mL of RNA*later* (Qiagen, www.qiagen.com) for 12 h at 4ºC and overnight incubation at -20°C (RNA*later*), c) pellet fixation in 1mL of TRIzol Reagent (Invitrogen, www.invitrogen.com) and storage at -80°C (TRIzol -80°C) for 24 h, and d) pellet fixation in 1 mL of TRIzol Reagent for immediate extraction of total RNA (TRIzol) (see Table 1 and Table S1).

Tissue samples from gonads and digestive tract were dissected out of the animals, flash frozen in liquid nitrogen and storage at -80°C until processed. Tissue extraction was always performed with sterilized razor blades and forceps rinsed with RNaseAWAY (Sigma Aldrich, www.sigmaaldrich.com) to avoid RNA degradation.

RNA extraction

Besides the coelomocyte pellets, approximately 20 mg of gonad tissue or esophagus (the whole length) were used for extraction of total RNA. For samples preserved in liquid nitrogen and stored at -80ºC, two different methods of extraction were tested for best suitability in echinoderm samples: a) for direct extraction of poly(+A) mRNA we used the Dynabeads® mRNA DIRECT™ Kit (Invitrogen) following the manufacturer's instructions and b) for total RNA extraction we used an optimized TRIzol protocol. Due to the high viscosity of the tissue and cell samples, the direct mRNA extraction could not be successfully accomplished, and RNA extraction was always performed using TRIzol.

Either fresh or frozen tissues were homogenised with micropestles in 1 mL of TRIzol. One BCP (1-bromo-3-chloropropane) extraction was performed using 0.2 mL, followed by precipitation in 0.5 mL of isopropanol plus 1µL of RNaseOUT (Invitrogen), and overnight incubation at -20°C. Total RNA was then precipitated and pelleted using a 15 minutes centrifugation (16,000 g) at 4ºC, then the pellet washed twice in 75% ethanol with previous centrifugations (16,000 g) for 5 minutes at 4ºC and, re-dissolved in 55 µL RNase-free water plus 1uL of RNaseOUT. In order to avoid RNA degradation, the whole extraction protocol was developed on ice.

Integrity of total RNA was initially evaluated by visualising the 28S rRNA and 18S rRNA bands into a 1% agarose gel in 1x TAE Buffer. In addition, RNA has an absorbance maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm and 260 and 230 nm has been used to assess the RNA purity. An A260⁄230 ratio has been also used to estimate the presence of contaminants while A260⁄280 ratio was used to estimate the purity of RNA (Riesgo *et al.*

2012b). Absorbance ratios A260/230 and A260/280 and concentration of our extractions were assessed in a Hellma Spectrophotometer (Hellma Analytics). An RNA sample is considered "pure" when values for the A260⁄280 and A260⁄230 are between 1.8 and 2.2, and concentration over 200 ng/µL is considered acceptable, according to the manufacturer's instructions of the kit used for isolation of mRNA (TruSeq RNA sample prep kit from Illumina Inc.). RNA extractions were finally run in an Agilent 2100 Bioanalyzer (Agilent Technologies) at the Scientific and Technical Services of the University of Barcelona for quality measurements. RNA integrity was measured using the RIN software algorithm, which allows for the classification of eukaryotic total RNA, based on a numbering system from 1 to 10 (RIN value), with 1 being the most degraded profile and 10 being the most intact. RIN values over 8 were considered non-degraded usable RNA extractions.

Statistical analyses

The total number of samples consisted of 41 extractions from three different tissue types and four different treatments for coelomocytes (Table 1).

We firstly investigated whether measures of RNA quality, absorbance ratios (A260/230 and A260/280) and RIN value variables were correlated in our data. Since RIN did not follow a normal distribution (Shapiro-Wilk test: $W = 0.7593$, $p < 0.001$), even after we applied the logarithmic transformation to the original values (Shapiro-Wilk test: $W = 0.6169$, $p < 0.001$), a non-parametric Spearman's correlation coefficient was applied.

To test for differences in the RNA concentration of different tissues and treatments we initially used a two-way ANOVA, after confirming normality and homoscedasticity of the

dependent variable. One-way ANOVA was also applied to evaluate the effect of the treatments on the RNA concentration from coelomocytes. We also investigated if RNA quality, measured as RIN values, depended on either the tissue type or the different treatments (here considering only the coelomocytes) by Kruskal-Wallis non-parametric analyses.

Distribution of the variables RIN and RNA concentration were graphically represented in boxplots for different tissues and treatments. Statistical analyses and boxplots were performed using the software “R v. 2.15.2”.

Results

The Spearman's coefficients did not detect correlation between RNA quality variables based on absorbance ratios (A260/280 and A260/230) and RIN values (ρ=0.498 and ρ=0.7496, respectively; p<0.001). In some samples with A260/280 and A260/230 ratios over 2.0, we observed RIN values lower than 8 (see examples in Fig. 1) (Table S1, Supporting information), showing that the 28S and 18S peaks were close to intact but the fast region (mRNA) and the 5S were completely degraded (Fig. 2) .

We did not detect significant differences in RNA quantity and quality for the three tissues analysed. RNA concentration was not significantly different among tissues (ANOVA, F=7.4, p > 0.05). Both digestive and gonad tissues presented good values of concentration and RIN for further mRNA isolation. RIN values did not either display significant differences between the three tissue types (Kruskal-Wallis , H= 1.6549, p > 0.05) (Fig. 3). The high variability of RIN values in coelomocytes was mostly due to the different preservation treatments applied (see below).

For coelomocytes, we observed significant differences in both RNA quality, based on RIN values (Kruskal-Wallis, H=18.45, df=3, p<0.001), and quantity (ANOVA, F=5.548, p=0.004) depending on the treatment applied (Fig. 4). Flash freezing of coelomocytes provided high RNA concentrations but degraded RNA for most samples. RIN values for flash-frozen samples were between 1 and 7.2, which were significantly lower than those obtained from "TRIzol" and "TRIzol -80ºC" treatments (see Fig. 4 and Table S1). Fixation in RNA*later* resulted in variable values of RIN (from 5.6 to 9.0, with median about 8). The concentration of RNA*later* samples was significantly lower than that of all the other treatments (from 22 ng/uL to 70 ng/uL) (Fig. 4 and Table S1). We did not observe significant differences in RNA quality and quantity between "TRIzol" and "TRIzol -80ºC" treatments (Kruskal-Wallis, H=16.32, p>0.05; ANOVA, H=1.119, p>0.05 for quality and quantity, respectively) but there was a wider variability in RNA concentration values in the "TRIzol -80ºC" treatment. In agarose gels, the quality of RNA samples varied greatly among treatments (Fig. 2). While samples flash-frozen in liquid nitrogen presented degraded RNA with no visible 18S and 28S bands and a wide smear in the fast region, the samples preserved in TRIzol (whether or not conserved at -80ºC) showed the sharpest and cleanest bands for 18S and 28S. For the RNA*later* preserved samples, the quantity was so low (always below 70 ng/µL) that hampered the visualization of the bands using standard agarose electrophoresis (Fig. 2).

Discussion

Assessment of RNA quality can be performed measuring different features: overall degradation through visualization of 18S and 28S bands in a standard agarose gel, A260/280 and A260/230 ratios, and estimation of the RIN value (Gayral *et al.* 2011; Hillyard & Clark

2012; Riesgo *et al*. 2012b). In our results, the most efficient strategies for assessing the RNA integrity were the electrophoresis in agarose gels and the estimation of the RIN value using Agilent Bioanalyzer chips. For coelomocytes, there were no consistent correlations between the RNA integrity and the A260/280 and A260/230 ratios. This could be due to the different stability of the RNAs, being the ribosomal RNA more stable than the mRNA (Houseley & Tollervey 2009). Then, even though the mRNA might be degraded, the A260/280 could still render values around 2 due to the intact nature of the ribosomal RNA. If working with coelomocytes, it would be important to assess the RNA integrity using bioanalyzer profiles, since in this case the bioanalyzer profile would show degradation in the fast and 5S regions.

All tissues extracted during the study contained enough RNA amounts to further construct cDNA libraries for Next-Generation sequencing technologies. However, the preservation method needed to be adjusted in the case of coelomocytes in order to obtain good-quality RNA. Undegraded RNA was successfully extracted with TRIzol from flash-frozen digestive and gonad tissues, as occurred in other flash-frozen solid tissues or biological fluids of other non-model invertebrates (Santiago-Vázquez *et al*. 2006; Pinsino *et al*. 2008, Gayral *et al*. 2011; Simister *et al.* 2011; Hillyard & Clark 2012; Riesgo *et al*. 2012a, b). However, that was not the case for coelomocytes of *Arbacia lixula*. Flash-frozen coelomocytes rendered considerable RNA amounts with very low quality (estimated using RIN values and observed also in agarose gels). Coelomocytes are cells containing a rich selection of lysosomal enzymes (Stabili *et al* 1994; Haug *et al* 2002), among which RNases may be present. Therefore, cell lysis should be avoided to prevent RNA degradation by the echinoderm own RNases. During sample freezing, cell lysis can occur when the produced micro-crystals break the cellular membranes; hence, although flash-freezing is advisable for solid tissues in

general, it should be avoided when dealing with coelomic fluids unless an RNase inhibitor is added to the fluid. One solution for preventing cell lysis is the use of imidazole, which is commonly added to the anticoagulant buffer used for withdrawal of coelomic fluid in other echinoderms (Gross *et al.* 1999). Imidazole inhibits the activity of lysosomic enzymes (such as lysozyme) (Shinitzky *et al*. 1966), and therefore cell lysis is prevented.

Another solution equally effective in maintaining the RNA integrity is the use of TRIzol reagent in freshly collected cells, since it contains high concentrations of guanidine thiocyanate and acid phenol to inhibit RNase activity. The advantages of using TRIzol rely on the absence of other foreign substances that could interpose in the subsequent procedures.

RNA extraction from fresh tissues is used in many cases with success (Gross *et al*. 1999; Matranga *et al.* 2000; Nair *et al.* 2005; Pinsino *et al*. 2008), but sometimes, field or laboratory conditions do not allow for direct extraction upon collection. We demonstrated here that the best option for preservation and storage of RNA from coelomocytes, when the direct extraction could not be performed, is the combination of preservation in TRIzol and storage at -80ºC for long periods. In this case, large variability in the concentration of RNA recovered should be taken into account.

When working in the field, sometimes freezers are not even available, and another strategy of preservation might be required. RNA*later* has been proved to be a reliable preservative for RNA in a wide array of tissues (Gayral *et al*., 2011; Hillyard & Clark 2012), although unadvised for animal cells and fluids. Unexpectedly, in fluids such as urine, and sperm, the addition of RNA*later* to the cell pellet improved the RNA yield (Medeiros *et al*. 2003; Das *et al*. 2010), thus providing a promising perspective for coelomocyte preservation. However, coelomocyte pellets preserved in RNA*later* yielded limited amounts of RNA, similar to the results obtained for human blood (Weber *et al.* 2010). Therefore, when large amounts of

RNA (larger than 200 ng/µL) are needed, the use of RNA*later* as a preservative is unadvised when dealing with fluids containing phagocytic cells.

In conclusion, flash-freezing is an adequate method of RNA preservation for solid tissues in echinoderms. For coelomocytes, extraction of freshly collected cell pellets rendered the best results in terms of quantity and quality of RNA. If direct extraction cannot be performed, the most reliable preservation method is the immersion of the coelomocyte cell pellets in TRIzol and subsequent storage at -80ºC.

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Figure legends

Fig. 1. Agilent Bioanalyzer profiles. Example of profiles showing RIN value and absorbance ratios for four different treatments in coelomocytes: liquid nitrogen (LN_2), RNA*later*, TRIzol at -80ºC, TRIzol from fresh cells.

Fig. 2. Agarose gels in 1x TAE buffer. RNA extractions from coelomocytes for four different treatments in coelomocytes: liquid nitrogen (LN_2), RNA *later*, TRIzol at -80ºC, TRIzol from fresh cells. 28S rRNA, 18S rRNA, 5S rRNA and small RNAs are intact for some treatments/samples .

Fig. 3. Boxplots of RIN value and RNA concentration (ng/µL) attributed to the different tissues (coelomocytes, digestive and gonad).

Fig. 4. Boxplots of RIN value and RNA concentration (ng/µL) attributed to the different treatments of coelomocytes: liquid nitrogen (LN_2), RNA*later*, TRIzol at -80ºC and TRIzol from fresh cells.

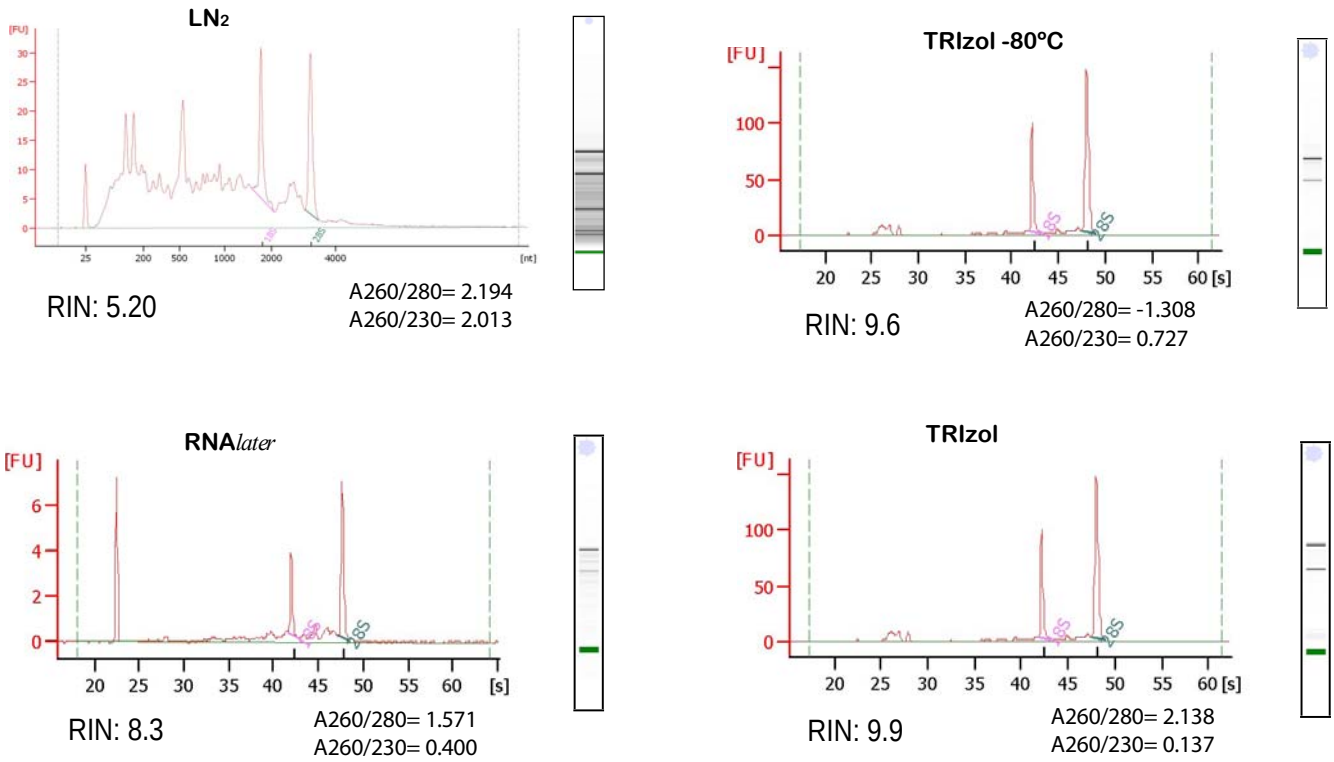
480

481 Table 1. Tissue type, preservation treatment, number of samples analysed (*n*), RIN value,

482 RNA concentration (ng/µL) and profile features.

Tissue	***Preservation***	***n***	***RIN***	***Concentration***	**Profile features**
Coelomocytes	LN_2	10	1.0- 7.2	210- 1,524	Good quantity but very low quality. Degraded RNA
Coelomocytes	RNA*later*	6	5.6- 9.0	22- 70	Very low quantity and variable quality. Insufficient mRNA for cDNA library construction
Coelomocytes	TRIzol -80°C	6	9.1- 9.7	34- 1,650	High variability in quantity but good quality
Coelomocytes	TRIzol	10	8.3- 10	220- 1,022	Good quantity and quality
Gonad	LN_2	6	8.3- 10	388- 1,680	Good quantity and quality
Digestive	LN_2	3	8.6- 9.4	424- 852	Good quantity and quality

483 Fig. 1



484

485

486 Fig.2

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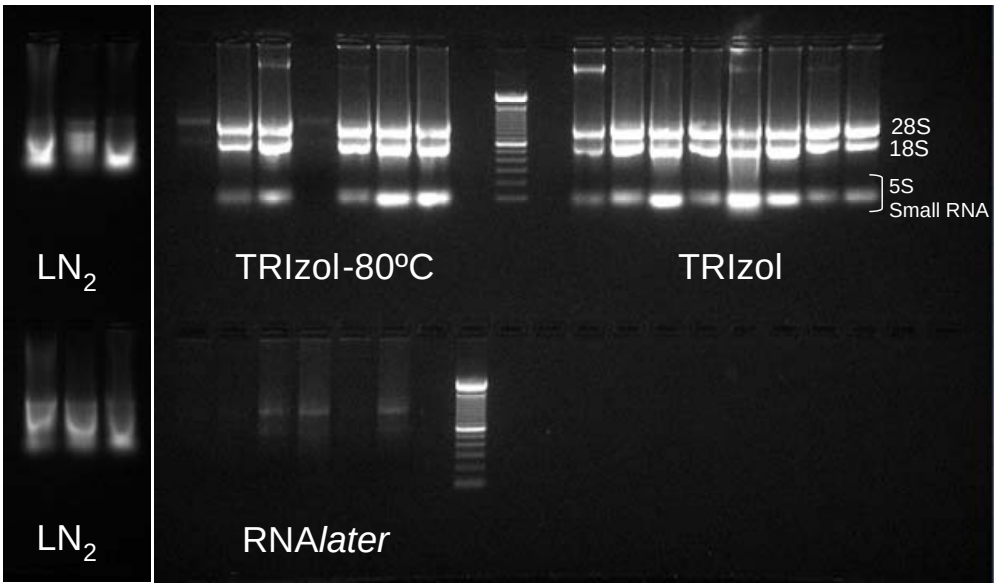


496

Fig. 3

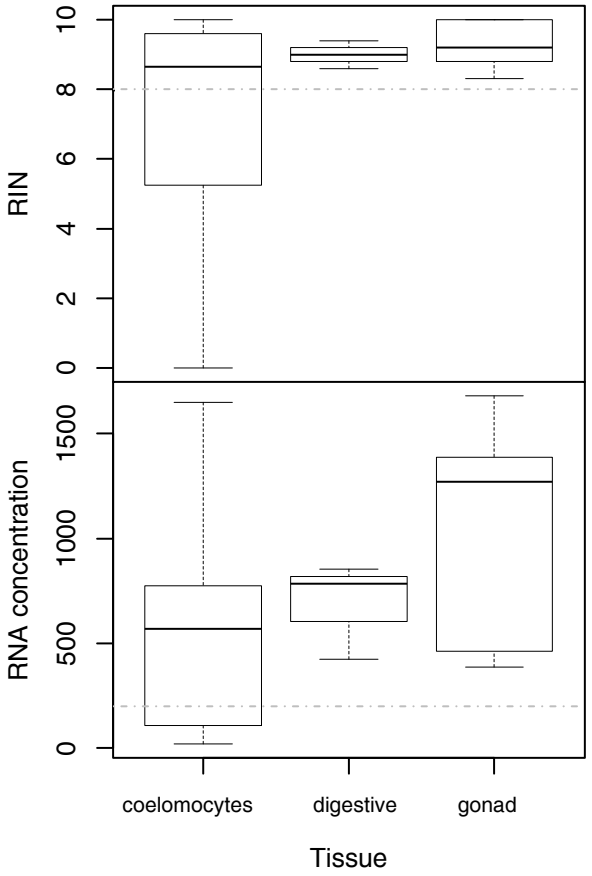
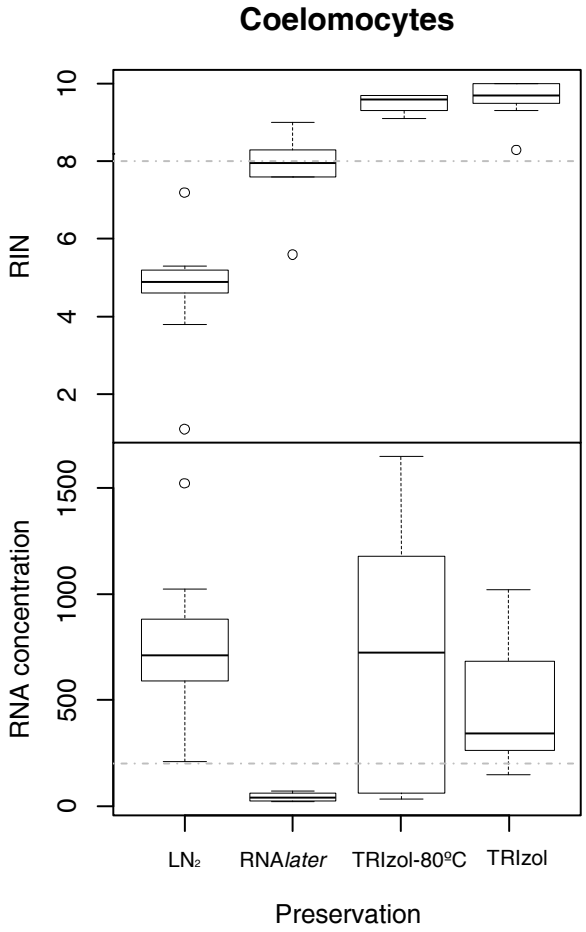

RIN
0
2
4
6
8
10
RNA concentration
0
500
1000
1500
coelomocytes
digestive
gonad
Tissue

Fig. 4


Coelomocytes
RIN
0
2
4
6
8
10
RNA concentration
0
500
1000
1500
LN2
RNAlater
TRIzol-80ºC
TRIzol
Preservation